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Only Two of the Five Zinc Fingers of the Eukaryotic Transcriptional Repressor PRDI-BF1 Are Required for Sequence-Specific DNA Binding

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The eukaryotic transcriptional repressor PRDI-BF1 contains five zinc fingers of the C_2H_2 type, and the protein binds specifically to PRDI, a 14-bp regulatory element of the beta interferon gene promoter. We have investigated the amino acid sequence requirements for specific binding to PRDI and found that the five zinc fingers and a short stretch of amino acids N terminal to the first finger are necessary and sufficient for PRDI-specific binding. The contribution of individual zinc fingers to DNA binding was investigated by inserting them in various combinations into another zinc finger-containing DNA-binding protein whose own fingers had been removed. We found that insertion of PRDI-BF1 zinc fingers 1 and 2 confer PRDI-binding activity on the recipient protein. In contrast, the insertion of PRDI-BF1 zinc fingers 2 through 5, the insertion of zinc finger 1 or 2 alone, and the insertion of zinc fingers 1 and 2 in reverse order did not confer PRDI-binding activity. We conclude that the first two PRDI-BF1 zinc fingers together are sufficient for the sequence-specific recognition of PRDI.

Structure-function analyses of transcriptional regulatory proteins have identified a number of discrete structural domains that mediate specific DNA binding (see reference 16 for a review). The zinc finger motif of the Cys-Cys–His-His (C_2H_2) type was first identified in the sequence of *Xenopus laevis* transcription factor IIIA (TFIIIA) (48; reviewed in references 5 and 9). Subsequently, zinc fingers were found in many proteins in diverse eukaryotic species ranging from yeast cells to humans (for example, see references 34, 40, and 46). The zinc finger motif is characterized by the 30-amino-acid consensus sequence Tyr/Phe-X-Cys-$X_{2/4}$-Cys-X_3-Phe-X_5-Leu-X_2-His-X_3-His-Thr-Gly-Glu-Lys-Pro, although many variations occur. Some proteins have only one zinc finger (1, 24), and others have up to 19 contiguous zinc fingers (3, 7), while others have zinc fingers or groups of zinc fingers that are separated from each other within the protein (11, 28, 38, 41). At present, only a small fraction of zinc finger-containing proteins have been shown to bind to DNA or RNA.

The first evidence that zinc fingers are involved in DNA binding came from studies with TFIIIA. Proteolytic fragments of TFIIIA containing virtually only zinc fingers were found to bind to DNA (44, 50). Studies of the interactions between TFIIIA and the internal control region of the 5S RNA gene revealed nine points of contact separated by 5-bp intervals along the DNA (10, 39). These contact points were proposed as sites of interaction with each of the nine zinc fingers of TFIIIA. Subsequently, the DNA-binding domains of other zinc finger-containing proteins have been localized to peptide fragments which include zinc fingers (11, 18). In some proteins, DNA binding has been shown to require zinc (15, 17, 26). The sequence-specific DNA-binding potentials of zinc fingers were directly assessed by using isolated zinc finger peptides. A synthetic single zinc finger was shown to fold into a zinc-dependent conformation yet was unable to bind specifically to DNA (12). However, an 89-residue peptide containing the three zinc fingers of the yeast transcriptional regulatory protein SWI5 was shown to footprint on the HO promoter (29).

A model for the three-dimensional folding of zinc fingers proposed by Berg (4) was based on the similarity of zinc fingers to certain metalloenzymes. According to this model, zinc fingers are independent domains in which the invariant two cysteine and two histidine residues hold a zinc atom in tetrahedral geometry. The cysteines are part of a β sheet, while the histidines are on one face of an α helix that lies in the major groove of the DNA. Specific contacts are made between the amino acids on the face of the α helix opposite the zinc ion and DNA nucleotides. A very similar model was suggested by Gibson et al. (14). The essential features of this model have been confirmed by extended X-ray absorption fine structure (EXAFS) (8) and nuclear magnetic resonance (22, 23, 33, 36) studies on synthetic zinc fingers (30) and more recently by the determination of the three-dimensional structure by X-ray crystallography (37). The multiple zinc fingers found in many zinc finger proteins appear to form a repeating structure when bound to DNA. Three base pairs of DNA are contacted by each finger, and there is no spacing between the 3-bp binding sites.

PRDI-BF1 is a transcriptional repressor that contains five zinc fingers and specifically binds to the PRDI element of the beta interferon promoter (19). We used the λgt11 expression system (51) in conjunction with an in situ binding assay (42, 43, 49) to study the contribution of each of the PRDI-BF1 zinc fingers to the sequence-specific recognition of PRDI. We found that the zinc fingers of PRDI-BF1 do not make equal contributions to specific binding. PRDI recognition requires the first zinc finger of PRDI-BF1 but does not require the last three. Furthermore, the first two zinc fingers together are sufficient to confer PRDI-binding specificity on another protein. These two zinc fingers together constitute the minimal functional PRDI recognition domain, since neither zinc finger by itself exhibits specific DNA-binding activity.

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MATERIALS AND METHODS

Packaging of mutants. Mutant PRDI-BF1 and PRDII-BF1 cDNAs, as well as constructs in pΞ (Table 1), were gel purified in low-melting-point agarose on an *Eco*RI fragment. They were ligated to λgt11 phage arms (Stratagene) in a 5-μl reaction mixture at 10°C overnight. A portion of the ligation mix was then packaged (Gigapack Gold; Stratagene) and plated for assay. Both sense and antisense orientations of insert were represented on each plate. For phage that were positive for binding to PRDI or PRDII, plaque purification was performed. On some phage that were negative for binding, individual plaques were purified and assayed to select for the sense orientation of the insert.

Binding of mutants in situ. λgt11 phage were plated, overlaid with nitrocellulose filters, and induced for fusion protein production as described by Young and Davis (51). Filters were then subjected to denaturation-renaturation and incubated with radiolabeled DNA probes according to the method of Vinson et al. (49). A buffer containing 12 mM Tris (7.9), 40 mM KCl, 0.12 mM EDTA, 30 μM $ZnSO_4$, and 400 μM β-mercaptoethanol was used for the denaturation, binding, and washing steps. Bovine serum albumin (fraction V) rather than dried milk was used as a blocking agent prior to and during the binding reaction. All binding reactions were carried out at 4°C overnight. Washes were performed at 4°C for 20 min with several changes of buffer. Probes were made from oligonucleotides which were ligated to 200- to 500-bp lengths and then radiolabeled by nick translation. Binding reactions contained 50 ng of probe and 2 μg of nonspecific calf thymus DNA per ml. Equal counts of the different probes were added to the binding reactions. The sequences of the oligonucleotides used as probes are as follows:

PRDI	GATCCAAGTGAAAGTGAAAGTGAAAGTGAGATC
PRDII	GATCTGTGGGAAATTCCGTGGGAAATTCCGGATC
octamer	GATCCATGCAAATAGATCT

Bacterial production of mutant protein. Several of the mutant proteins were also expressed in bacteria without fusion to LacZ and assayed for binding specificity on a Southwestern blot. Mutants were expressed off the phage T7 promoter (47). Protein was purified by the isolation of inclusion bodies, denaturation, and dialysis according to the method of Gaul et al. (13). Concentrations of protein were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. Between 5 and 30 μg of protein was electrophoresed on a protein gel, transferred to nitrocellulose, and hybridized with radiolabeled DNA probe in a Southwestern blot. The binding conditions were identical to those described above for the phage filters.

DNase I footprinting experiments. End-labeled DNA containing the PRDI site was incubated with ~50 ng of the indicated protein and treated with DNase I. The reaction products were isolated, denatured, and resolved by electrophoresis on a sequencing gel.

TABLE 1. Construction of mutants

Figure and part	Construction
2 (ii), 5C (i)	PRDI-BF1 *Bsm*I partial T4 blunted and fused to *Sty*I filled-in end
2 (iii)	PRDI-BF1 *Bsm*I complete T4 fused to *Sty*I filled-in end
2 (iv)	PRDI-BF1 *Bsm*I fragment replacing sequences downstream at second *Bsm*I site of 2 (ii); cut at *Stu*I site at 5′ end
4B (i), 5C (iii), 7 (i)	*Pst*I 14-mer (TGCACTGCAGTGCA) inserted at first PRDI-BF1 *Bsm*I site T4 blunted
4B (ii)	PRDI-BF1 *Bsm*I T4 blunt-ended fragment ligated in presence of several linkers (Fig. 4A)
5C (ii)	Upstream sequences of PRDII-BF1 to fusion point (asterisk in Fig. 5A) followed by *Pst*I 14-mer, PRDI-BF1 *Bsm*I T4 blunted to *Sty*I filled in followed by PRDII-BF1 sequences from *Aha*II filled in downstream
5C (ii)	PRDI-BF1 sequences downstream of *Sty*I filled-in end ligated to PRDII-BF1 upstream region at its *Aha*II filled-in site
5C (iv)	Same as 5C (ii) except sequences downstream of fingers remain PRDI-BF1
6C (i, iii, v, vi, vii, viii, ix, x)	Fingers ligated into pΞ on *Bsm*I-*Eag*I/*Hae*III and *Eag*I/*Hae*III-*Bsm*I fragments (Fig. 6A and B)
6C (ii), 8 (iii)	Synthesized PRDII-BF1 finger cloned into pΞ and then ligated to PRDII-BF1 at *Stu*I site in second finger
6C (iv)	PRDII-BF1 internal deletion from *Stu*I site to *Aha*II mung bean blunted end
7 (ii)	PRDI-BF1 *Bsm*I complete T4 blunted deletion with insertion of *Pst*I 14-mer
7 (iii)	PRDI-BF1 *Pst*I (in second finger) to *Sty*I filled-in fragment cloned into construct in 5C (i) at *Pst*I (in second finger) to *Hae*II (at pΞ finger insertion point)
7 (iv)	Same as 7 (iii) except PRDI-BF1 fragment inserted into construct 6C (v)
8 (ii)	*Bsm*I and downstream sequences from 8 (i) ligated into *Bsm*I site of 8 (iii), replacing its downstream sequences

RESULTS

The protein PRDI-BF1 contains five zinc fingers and binds specifically to the PRDI motif of the beta interferon promoter. PRDI-BF1 was initially isolated in a λgt11 screen as a partial protein fused to LacZ that binds to the PRDI element GAGAAGTGAAAGTG but not to PRDII, an adjacent element of the beta interferon promoter, GTGGG AAATTCC (19). The region of PRDI-BF1 containing the zinc fingers is shown in Fig. 1. All five zinc fingers match the commonly observed variant TFIIIA consensus, in which the two cysteines are separated by two residues rather than four. In addition, the fifth zinc finger has a cysteine in place of the second histidine. To investigate the role of the five zinc fingers in PRDI-specific binding, several mutants of PRDI-BF1 were constructed in the context of the original partial cDNA clone and packaged into λgt11 at the *Eco*RI cloning site. The recombinant phage thus encode a LacZ–PRDI-BF1 fusion protein. Phage were plated, induced for fusion protein production with isopropyl-β-D-thiogalactopyranoside (IPTG), and assayed in situ for binding activity to either a multimerized PRDI or PRDII DNA probe.

Initially, convenient restriction sites were used to delete either the last three zinc fingers or all five zinc fingers of PRDI-BF1. Figure 2 shows that wild-type PRDI-BF1 (part i) and a mutant lacking zinc fingers 3 through 5 (part ii) both bind to the PRDI probe and not to the PRDII probe, whereas a mutant lacking all five zinc fingers (part iii) does not bind to either probe. Furthermore, a mutant containing two tandem copies of the first two zinc fingers and 65 residues of PRDI-BF1 N terminal to them (part iv) still binds to PRDI.

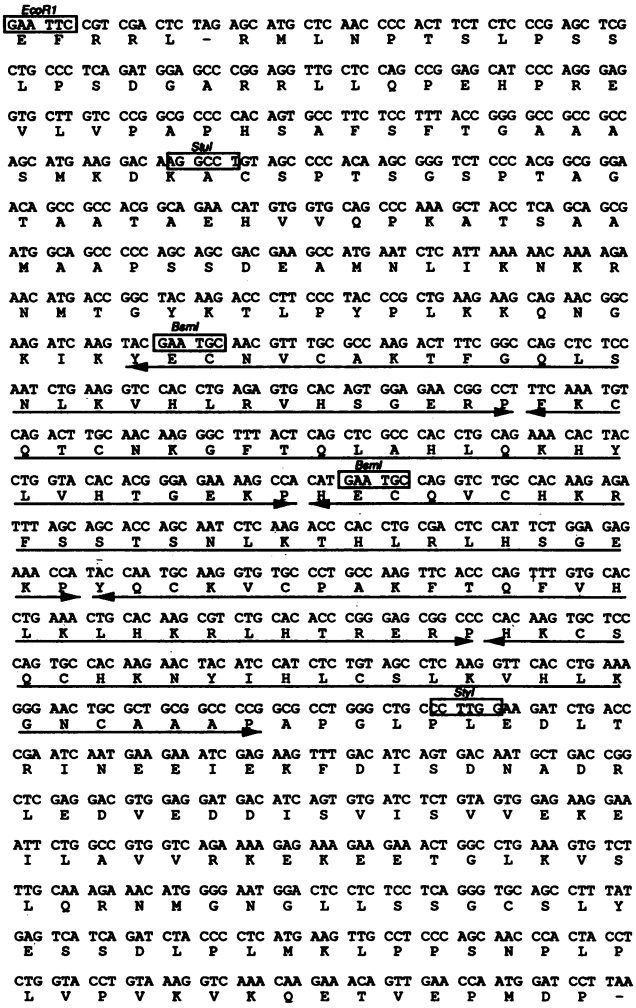


FIG. 1. DNA sequence of the partial PRDI-BF1 cDNA clone. The nucleotide sequence of the partial PRDI-BF1 cDNA, restriction sites, and the predicted partial PRDI-BF1 protein sequence are shown. The five zinc fingers are denoted by arrows under the sequence. The protein was expressed as a fusion to LacZ at the *Eco*RI site at the 5′ end. Although a TAG stop codon is present in the adaptor sequence between LacZ and PRDI-BF1, all studies were made in a suppressor bacterial strain allowing read-through translation.

Thus, the last three zinc fingers and many nonfinger regions of PRDI-BF1 are not necessary for PRDI-specific DNA binding.

Large amounts of wild-type PRDI-BF1 and the mutants lacking the last three or all five zinc fingers were synthesized in bacteria without fusion to LacZ on a T7 expression vector (47). The proteins were assayed for binding to PRDI and PRDII on a Southwestern (DNA-protein) blot (27). When excess quantities of protein are used in this assay, the amount of probe bound by the nitrocellulose-immobilized proteins should be proportional to the binding constants of the proteins. The fingerless mutant protein did not detectably bind to either the PRDI or the PRDII probe on a Southwestern blot, whereas the other two proteins bound to the PRDI probe with at least 100-fold-higher affinity than to the PRDII probe (data not shown). Thus, the binding affinities of the wild-type and mutant PRDI-BF1 proteins determined by Southwestern blotting correlate well with the binding behavior exhibited by those proteins as fusions to LacZ in λgt11 phage plaques in situ.

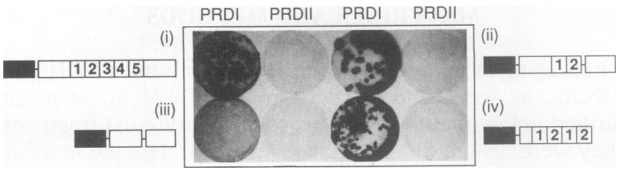


FIG. 2. PRDI-BF1 zinc fingers 3 through 5 are not necessary for PRDI binding. In situ binding of recombinant λgt11 phage to PRDI and PRDII probes is shown. Black boxes denote LacZ sequences, and white boxes denote PRDI-BF1 sequences. The five zinc fingers are numbered. (i) Wild-type PRDI-BF1; (ii) deletion of zinc fingers 3 through 5; (iii) deletion of zinc fingers 1 through 5; (iv) 65 residues of PRDI-BF1 (encoded downstream of the *Stu*I site) fused to tandem copies of the first two zinc fingers (see Materials and Methods for descriptions of the mutant clones).

This conclusion was confirmed by DNase I footprinting experiments, as shown in Fig. 3. The PRDI binding site was protected from DNase I digestion by a protein containing all five zinc fingers (lane 2) and by a protein containing only the first two fingers (lane 3). As expected, no footprint was observed when all five zinc fingers were removed. Thus, the same conclusions can be reached on the basis of the plaque assay, the preparative Southwestern blots, and DNase I footprinting experiments.

In order to determine whether a LacZ fusion protein containing only the first two PRDI-BF1 zinc fingers can bind to PRDI, it was necessary to clone a fragment of the PRDI-BF1 cDNA encoding only those zinc fingers. Excision of the first two zinc fingers on a *Bsm*I restriction fragment removed the first four residues from the first zinc finger. To replace these residues, a *Pst*I 14-mer linker was ligated to the

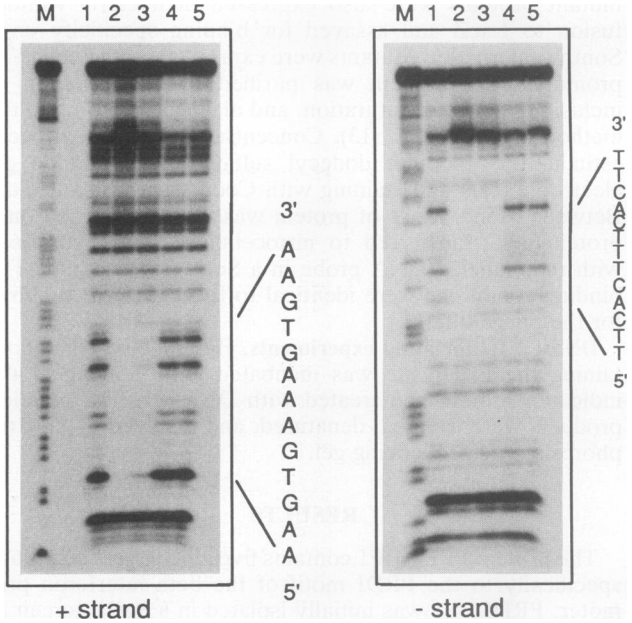


FIG. 3. DNase footprinting experiments with wild-type and mutant PRDI-BF1. Lanes: M, Maxam and Gilbert G+A sequence; 1, no protein; 2, wild-type PRDI-BF1; 3, PRDI-BF1Δ3-5; 4, PRDI-BF1Δ1-5; 5, no protein.

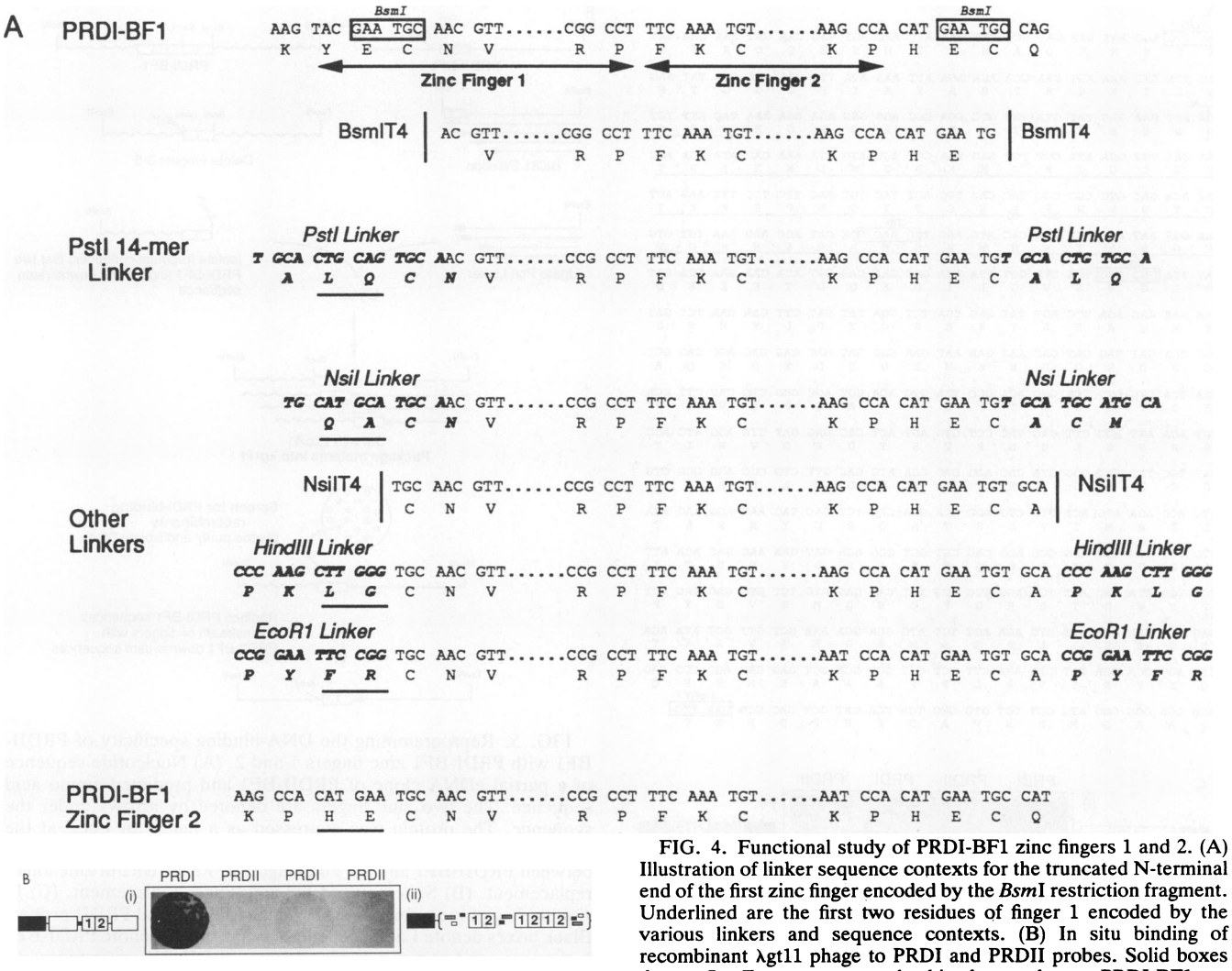


FIG. 4. Functional study of PRDI-BF1 zinc fingers 1 and 2. (A) Illustration of linker sequence contexts for the truncated N-terminal end of the first zinc finger encoded by the *Bsm*I restriction fragment. Underlined are the first two residues of finger 1 encoded by the various linkers and sequence contexts. (B) In situ binding of recombinant λgt11 phage to PRDI and PRDII probes. Solid boxes denote LacZ sequences, and white boxes denote PRDI-BF1 sequences. (i) *Pst*I 14-mer linker at the N-terminal end of the first zinc finger in the zinc finger 3 through 5 deletion; (ii) sample of LacZ fusion proteins resulting from the ligation products of linkers and the *Bsm*I restriction fragment encoding the first two PRDI-BF1 zinc fingers.

5′ end of the restriction fragment encoding the truncated zinc finger. This linker perfectly recreates the third and fourth residues of the first zinc finger and maintains a hydrophobic residue at the first position (Fig. 4A). This alteration of the first zinc finger had no noticeable effect on its activity. Figure 4B shows that a mutant PRDI-BF1 lacking zinc fingers 3 through 5 and having the *Pst*I linker N terminal to the truncated first zinc finger (part i) binds specifically to PRDI.

Single and tandem repeats of the *Bsm*I fragment encoding the first two zinc fingers of PRDI-BF1 were ligated directly onto *lacZ* with the *Pst*I linker at their 5′ ends. In addition, other linkers were added to create many different sequence contexts for the zinc fingers (Fig. 4A). When pools of these clones were ligated and assayed for DNA-binding activity in situ, no PRDI-binding activity was observed. Many recombinant phage were screened to ensure the representation of diverse linker combinations. Figure 4B, part ii, shows a representative filter of phage. This result indicates that the first two zinc fingers of PRDI-BF1 are not sufficient for binding to PRDI. The zinc fingers in conjunction with 65 N-terminal residues, however, are sufficient (Fig. 2, part iv). The PRDI-BF1 region immediately N terminal to the zinc fingers is quite basic (Fig. 1). This region may therefore participate directly in specific or nonspecific interaction with DNA or, alternatively, may contribute to the structural integrity of the zinc fingers.

Another zinc finger-containing protein, PRDII-BF1, has a sequence specificity very different from that of PRDI-BF1. PRDII-BF1 was initially isolated as a partial protein fused to LacZ (11). This protein, which has two zinc fingers and an adjacent N-terminal region that is highly basic (Fig. 5A), binds to PRDII and not to PRDI. If the first two zinc fingers of PRDI-BF1 are sufficient to determine the specificity of DNA binding yet not sufficient for DNA binding per se, it should be possible to reprogram the DNA-binding specificity of PRDII-BF1 by replacing its zinc fingers with the first two PRDI-BF1 zinc fingers. Figure 5B illustrates the scheme employed for the zinc finger replacement. Initially, a *Bal* 31 deletion series was made in the PRDII-BF1 cDNA to remove sequences encoding its zinc fingers while retaining variable amounts of sequences encoding the N-terminal basic region.

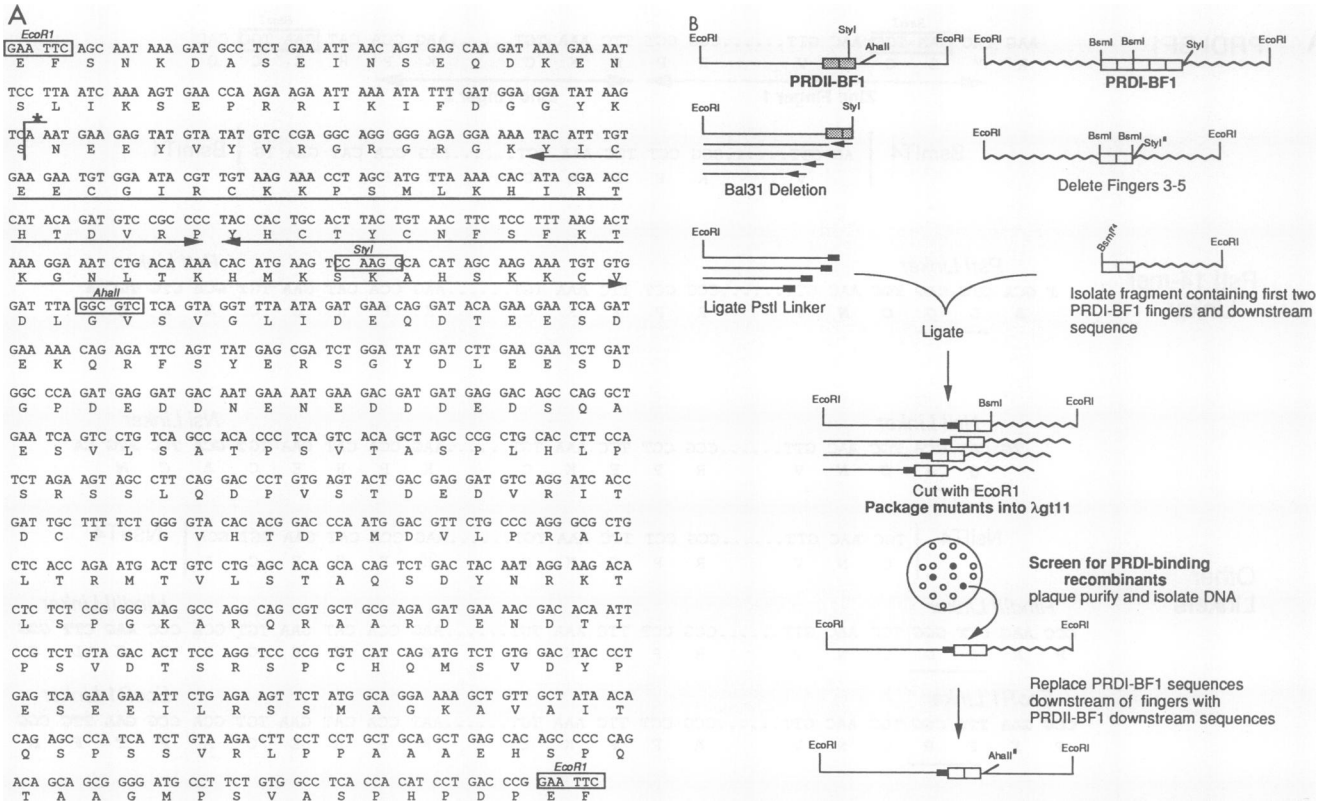
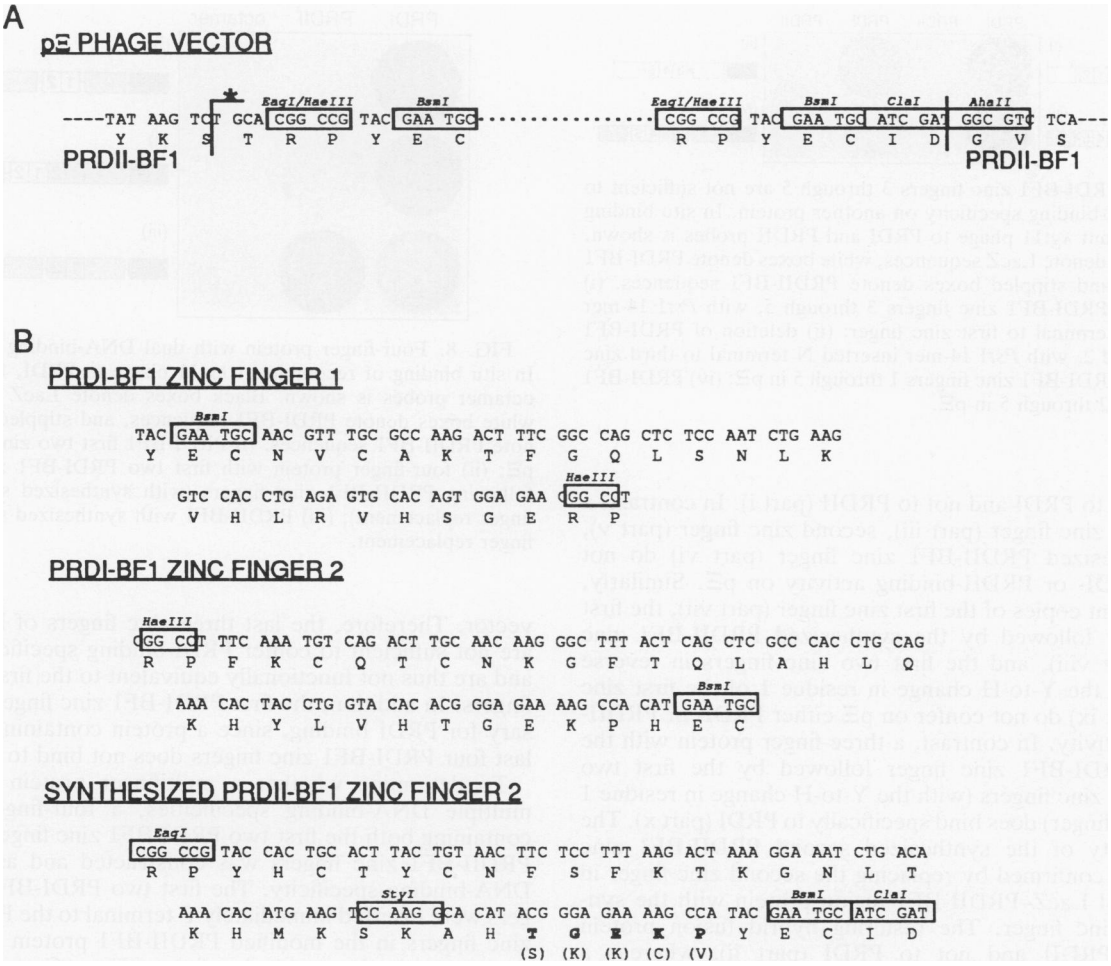


FIG. 5. Reprogramming the DNA-binding specificity of PRDII-BF1 with PRDI-BF1 zinc fingers 1 and 2. (A) Nucleotide sequence of a partial cDNA clone of PRDII-BF1 and predicted amino acid sequence. The two zinc fingers are denoted by arrows under the sequence. The protein was expressed as a fusion to LacZ at the *Eco*RI site at its 5′ end. The asterisk denotes the fusion point between PRDII-BF1 and the zinc fingers of PRDI-BF1 in zinc finger replacement. (B) Scheme used for zinc finger replacement. (C) In situ binding of recombinant λgt11 phage to PRDI and PRDII probes. Black boxes denote LacZ sequences, white boxes denote PRDI-BF1 sequences, and stippled boxes denote PRDII-BF1 sequences. (i) Deletion of PRDI-BF1 zinc fingers 3 through 5; (ii) PRDI-BF1 first two zinc fingers, with *Pst*I 14-mer at their N-terminal end, cloned into fingerless PRDII-BF1; (iii) deletion of PRDI-BF1 zinc fingers 3 through 5, with *Pst*I 14-mer at the N-terminal end of the first zinc finger; (iv) PRDI-BF1 zinc fingers 1 and 2 and C-terminal regions fused to the PRDII-BF1 N-terminal region, with the *Pst*I 14-mer inserted N terminal to the first zinc finger; (v) PRDII-BF1; (vi) region of PRDI-BF1 C terminal to its zinc fingers replacing region of PRDII-BF1 C terminal to its zinc fingers.

This deletion series was then ligated to a fragment of the PRDI-BF1 cDNA encoding its first two zinc fingers and C-terminal residues. Once again, the *Pst*I 14-mer linker was inserted upstream of the PRDI-BF1 cDNA *Bsm*I site to replace the truncated residues of the first PRDI-BF1 zinc finger. The resulting hybrid cDNA clones were ligated into λgt11 and screened in situ for PRDI-binding activity. Two phage encoding PRDI-binding fusion proteins were isolated and were found to be identical. The fusion point between the PRDII-BF1 N-terminal region and the PRDI-BF1 zinc fingers maintained a positive charge immediately N terminal to the zinc fingers and is indicated in Fig. 5A by an asterisk above the PRDII-BF1 sequence. The zinc finger replacement was completed by replacing PRDI-BF1 cDNA sequences downstream of the zinc fingers with PRDII-BF1 cDNA sequences downstream of the *Aha*II restriction site.

Figure 5C shows that the first two zinc fingers of PRDI-BF1 confer PRDI-binding specificity on PRDII-BF1. Whereas the hybrid fusion proteins containing the zinc fingers of PRDII-BF1 bind to PRDII and not PRDI (parts v and vi), the fusion proteins containing the first two zinc fingers of PRDI-BF1 bind to PRDI and not to PRDII (parts i, ii, iii, and iv). The zinc finger replacement (Fig. 5C, part ii) resulted in deletion of the two PRDII-BF1 zinc fingers and an additional 14 N-terminal and 3 C-terminal residues. We conclude that the first two zinc fingers of PRDI-BF1 alone are sufficient to determine the sequence specificity of PRDI binding.

In order to determine the role of each of the first two PRDI-BF1 zinc fingers in the sequence-specific recognition of PRDI, we constructed a phage vector, pΞ, which allows individual zinc fingers and combinations of multiple zinc fingers to be inserted into identical protein contexts (Fig. 6A). The pΞ vector was made by inserting cloning linkers in the finger replacement recombinant λgt11 phage (Fig. 5C, part ii) in place of the sequences encoding the two PRDI-BF1 zinc fingers. These linkers allow DNA fragments encoding



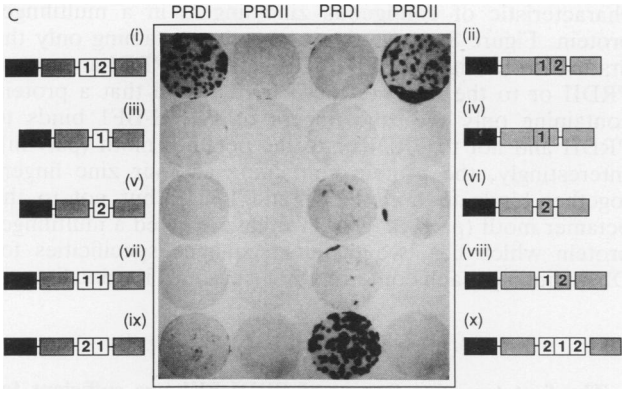


FIG. 6. Activities of zinc fingers in pΞ phage vector. (A) Sequence of pΞ phage vector zinc finger insertion point, showing cloning sites and the amino acid residues they encode. The upstream fusion to PRDII-BF1 sequences is indicated by the asterisk, while the downstream fusion to PRDII-BF1 occurs at the *Aha*II restriction site (Fig. 5A). (B) Sequences of the first two zinc fingers of PRDI-BF1 and the synthesized second zinc finger of PRDII-BF1. Changes in the 3′ linker sequence between the actual and synthesized zinc fingers are shown in parentheses below the sequence. (C) In situ binding of recombinant λgt11 phage to PRDI and PRDII probes. Black boxes denote LacZ sequences, white boxes denote PRDI-BF1 sequences, and stippled boxes denote PRDII-BF1 sequences. (i) PRDI-BF1 first two zinc fingers in pΞ; (ii) synthesized PRDII-BF1 second zinc finger replacement in PRDII-BF1; (iii) PRDI-BF1 first zinc finger in pΞ; (iv) partial second zinc finger deletion in PRDII-BF1; (v) PRDI-BF1 second zinc finger in pΞ; (vi) synthesized second zinc finger of PRDII-BF1 in pΞ; (vii) tandem copies of the first zinc finger of PRDI-BF1 in pΞ; (viii) first zinc finger of PRDI-BF1 followed by synthesized second zinc finger of PRDII-BF1 in pΞ; (ix) second zinc finger of PRDI-BF1 followed by first zinc finger of PRDI-BF1 in pΞ; (x) second zinc finger of PRDI-BF1 followed by first two zinc fingers of PRDI-BF1 in pΞ.

zinc fingers to be inserted into the protein, and they also encode part of the zinc finger consensus linker, arginine-proline, immediately upstream from the insertion sites. As illustrated in Fig. 6B, the first zinc finger of PRDI-BF1 (with no alterations in its first four residues) can be inserted into pΞ on a *Bsm*I-*Hae*III restriction fragment, while the second zinc finger of PRDI-BF1 can be inserted into pΞ on a *Hae*III-*Bsm*I restriction fragment. In addition, an oligonucleotide encoding the second zinc finger of PRDII-BF1 was synthesized with modifications in its C-terminal linker so that it could be inserted into pΞ on an *Eag*I-*Bsm*I restriction fragment and assayed along with the first two PRDI-BF1 zinc fingers. Furthermore, any zinc fingers can be combined in tandem while maintaining their integrity. The one exception is that when the first PRDI-BF1 zinc finger follows the second PRDI-BF1 zinc finger, its first residue is changed from tyrosine to histidine (Fig. 4A).

Figure 6C shows that when the first two zinc fingers of PRDI-BF1 were inserted into pΞ, the resulting fusion pro-

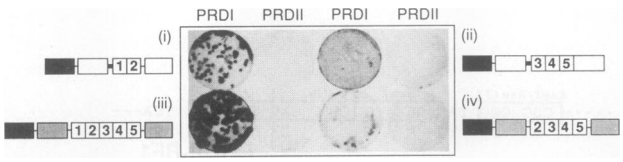


FIG. 7. PRDI-BF1 zinc fingers 3 through 5 are not sufficient to confer PRDI-binding specificity on another protein. In situ binding of recombinant λgt11 phage to PRDI and PRDII probes is shown. Black boxes denote LacZ sequences, white boxes denote PRDI-BF1 sequences, and stippled boxes denote PRDII-BF1 sequences. (i) Deletion of PRDI-BF1 zinc fingers 3 through 5, with *Pst*I 14-mer inserted N terminal to first zinc finger; (ii) deletion of PRDI-BF1 fingers 1 and 2, with *Pst*I 14-mer inserted N terminal to third zinc finger; (iii) PRDI-BF1 zinc fingers 1 through 5 in pΞ; (iv) PRDI-BF1 zinc fingers 2 through 5 in pΞ.

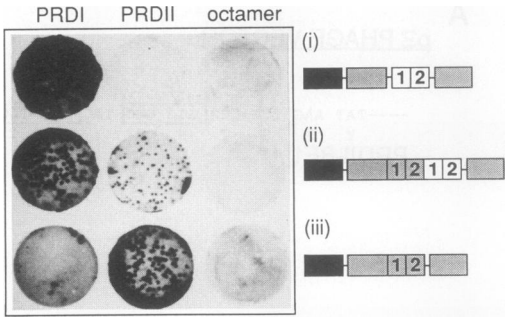


FIG. 8. Four-finger protein with dual DNA-binding specificity. In situ binding of recombinant λgt11 phage to PRDI, PRDII, and octamer probes is shown. Black boxes denote LacZ sequences, white boxes denote PRDI-BF1 sequences, and stippled boxes denote PRDII-BF1 sequences. (i) PRDI-BF1 first two zinc fingers in pΞ; (ii) four-finger protein with first two PRDI-BF1 zinc fingers following PRDII-BF1 zinc fingers (with synthesized second zinc finger replacement); (iii) PRDII-BF1 with synthesized second zinc finger replacement.

tein bound to PRDI and not to PRDII (part i). In contrast, a single first zinc finger (part iii), second zinc finger (part v), and synthesized PRDII-BF1 zinc finger (part vi) do not confer PRDI- or PRDII-binding activity on pΞ. Similarly, two adjacent copies of the first zinc finger (part vii), the first zinc finger followed by the synthesized PRDII-BF1 zinc finger (part viii), and the first two zinc fingers in reverse order with the Y-to-H change in residue 1 of the first zinc finger (part ix) do not confer on pΞ either PRDI- or PRDII-binding activity. In contrast, a three-finger protein with the second PRDI-BF1 zinc finger followed by the first two PRDI-BF1 zinc fingers (with the Y-to-H change in residue 1 of the first finger) does bind specifically to PRDI (part x). The functionality of the synthesized second PRDII-BF1 zinc finger was confirmed by replacing the second zinc finger in the original LacZ–PRDII-BF1 fusion protein with the synthesized zinc finger. The resulting hybrid fusion protein binds to PRDII and not to PRDI (part ii), whereas a LacZ–PRDII-BF1 protein containing a partial deletion in the second PRDII-BF1 zinc finger binds to neither probe (part iv). Some of these mutants were overproduced in bacteria on a T7 expression vector and assayed for binding on a Southwestern blot. In all cases, the binding behavior of the mutants in a Southwestern blot was identical to the binding behavior of the mutants in λgt11 phage in situ. We conclude that a pair of zinc fingers is the smallest unit capable of conferring either PRDI- or PRDII-binding specificity in the context of the pΞ vector.

The last three zinc fingers of PRDI-BF1, though not necessary for PRDI binding, might be functionally equivalent to the first two PRDI-BF1 zinc fingers. We tested for the ability of the last three zinc fingers to confer PRDI-binding specificity in two different protein contexts. In the first context, the *Bsm*I restriction fragment in the PRDI-BF1 cDNA clone was replaced with the *Pst*I 14-mer linker so that it encodes a fusion protein containing only the last three PRDI-BF1 zinc fingers. In this context, the *Pst*I 14-mer linker changes the four truncated residues of the third zinc finger from HECQ to LQCN. As shown in Fig. 7, whereas the first two PRDI-BF1 zinc fingers in this context confer PRDI-specific DNA binding (part i), the last three zinc fingers do not (part ii). In the second context, either all five PRDI-BF1 zinc fingers or only the last four were inserted into pΞ along with 7 C-terminal residues. The fusion protein containing all five zinc fingers specifically bound to PRDI (part iii), while the protein lacking only the first zinc finger did not (part iv). This result was corroborated by Southwestern blots using protein produced from the T7 expression vector. Therefore, the last three zinc fingers of PRDI-BF1 are not sufficient to confer PRDI-binding specificity on pΞ and are thus not functionally equivalent to the first two zinc fingers. In addition, the first PRDI-BF1 zinc finger is necessary for PRDI binding, since a protein containing only the last four PRDI-BF1 zinc fingers does not bind to PRDI.

To determine whether a multifinger protein can have multiple DNA-binding specificities, a four-finger protein containing both the first two PRDI-BF1 zinc fingers and the PRDII-BF1 zinc fingers was constructed and assayed for DNA-binding specificity. The first two PRDI-BF1 zinc fingers were inserted immediately C terminal to the PRDII-BF1 zinc fingers in the modified PRDII-BF1 protein containing the synthesized second zinc finger (Fig. 6C, part ii). The juxtaposed zinc finger pairs perfectly maintained the spacing characteristic of contiguous zinc fingers in a multifinger protein. Figure 8 shows that a protein containing only the first two zinc fingers of PRDI-BF1 binds to PRDI and not to PRDII or to the octamer motif (part i) and that a protein containing only the zinc fingers of PRDII-BF1 binds to PRDII and not to PRDI or to the octamer motif (part iii). Interestingly, the protein containing all four zinc fingers together binds to both PRDI and PRDII but not to the octamer motif (part ii). Thus, we have created a multifinger protein which has two distinct sequence specificities for DNA binding, each conferred by a subset of its zinc fingers.

DISCUSSION

The first two zinc fingers of PRDI-BF1 are sufficient for sequence-specific recognition of PRDI. Although a number of proteins containing multiple zinc fingers have been identified, the DNA sequences recognized by most of these proteins are not known. In addition, the question of whether the entire set of fingers is involved in recognizing a single DNA sequence or whether subsets of fingers from the same protein recognize different DNA sequences has not been answered. Initially, we demonstrated that the five zinc fingers of PRDI-BF1 can confer PRDI-binding specificity on another protein. We then showed that the first finger is necessary but not sufficient for PRDI binding and that fingers 3 through 5 are not required for binding to PRDI. Although the first zinc finger alone is not sufficient to confer PRDI-

binding specificity on another protein, the first two zinc fingers are sufficient. The zinc fingers in other multifinger proteins may also make unequal contributions to sequence-specific DNA binding. For example, the first zinc finger of TFIIIA is necessary for sequence-specific binding to the 5S RNA gene internal control region, yet the last three zinc fingers are not necessary for that interaction (50).

The protein contexts of zinc fingers influence their binding activities. Although the first two zinc fingers of PRDI-BF1 are sufficient to confer the specificity of DNA binding on another protein, they are insufficient to confer DNA binding. PRDI-BF1 residues outside of the zinc finger domains are also necessary. When the first two zinc fingers were fused to LacZ in a number of different protein contexts, no PRDI-binding activity was observed. When PRDI-BF1 residues N terminal to the zinc fingers were included, however, the resulting LacZ fusion protein did bind specifically to PRDI. Additional evidence that sequences N terminal to the zinc fingers are important for zinc finger activity comes from the zinc finger replacement experiment. In a pool of *Bal* 31 deletion mutants, only one fusion point between the zinc fingers of PRDI-BF1 and the N-terminal residues of PRDII-BF1 which rendered the zinc fingers functional was identified. The residues immediately adjacent to the zinc fingers are basic and might supply a nonspecific DNA-binding function. Alternatively, these nonfinger sequences may influence the structure of the zinc finger domains, ensuring that they are in the proper conformation. Thus, although zinc fingers are independently folding domains (12), the binding activities of the first two PRDI-BF1 zinc fingers depend very much on the protein context of the zinc fingers.

A pair of zinc fingers is a functional sequence recognition unit. We found that a pair of zinc fingers is the smallest unit capable of conferring the specificity of DNA binding on a fingerless protein. By constructing a phage vector into which several zinc fingers could be cloned in identical protein contexts, we were able to assay the activities of zinc fingers by themselves and in new combinations. Only the first two zinc fingers of PRDI-BF1 and the two zinc fingers of PRDII-BF1 in their original order were functional. It is unlikely that only one zinc finger is sufficient for determining the specificity of DNA binding, yet two zinc fingers are necessary to achieve sufficient nonspecific DNA-binding activity. Proteins containing the first two zinc fingers in reverse order, tandem copies of the first zinc finger, or the first zinc finger of PRDI-BF1 followed by the second zinc finger of PRDII-BF1 did not bind to either PRDI or PRDII. It is possible that both zinc fingers in the pair contribute to sequence-specific DNA binding by recognizing only a half site of DNA or that two zinc fingers together adopt a conformation suitable for sequence-specific DNA interaction.

As yet, there is no evidence that a single zinc finger is functional. Although some proteins contain only one zinc finger (1, 24) or an isolated single zinc finger (2, 11, 28, 35), it is still not known whether those single zinc fingers have a role in DNA recognition. Genetic studies have shown that both zinc fingers of the yeast factor ADR1 are necessary for its transcriptional activity in vivo (6). Mutations in either zinc finger disrupt ADR1 function, presumably by affecting DNA binding. More recently, Mardon and Page (25) identified a higher-order zinc finger repeat of the form $(ab)_n$ among the 13 zinc fingers in both mouse and human Zfy-2. Alternating zinc fingers were more similar to each other than to adjacent zinc fingers. Nietfeld et al. (31) analyzed 42 multifinger *Xenopus* clones and found many examples in which pairs of zinc fingers are a unit of repeat. It is possible that in these cases, as well as in PRDI-BF1, a pair of zinc fingers constitutes a functional DNA sequence recognition domain.

Recently, the three-dimensional structure of the three-zinc-finger protein Zif268 was reported (37). In this structure, the zinc finger residues 12, 15, and 18 are involved in sequence-specific interactions with three contiguous bases of DNA. On the basis of these and other observations, simple rules for the recognition of DNA by zinc fingers were proposed (21, 37). These rules involve interactions between guanine nucleotides and arginine and histidine residues at zinc finger positions 12, 15, and 18. By using these rules, the binding sites of several zinc finger proteins which recognize GC-rich sites were predicted. However, the PRDI-BF1 and PRDII-BF2 binding sites, which are not GC rich, do not conform to these rules. Both PRDI-BF1 and PRDII-BF2 proteins have very few arginine and histidine residues at zinc finger positions 12, 15, and 18, and they recognize binding sites that are relatively AT rich. In addition, methylation interference studies suggest that PRDI-BF1 contacts the PRDI site over at least seven nucleotides and therefore would require contacts with at least three zinc fingers. We have shown, however, that only two zinc fingers are sufficient for sequence-specific binding to PRDI. It is possible that the two-finger PRDI-BF1 mutant contacts fewer nucleotides than the wild-type protein, despite a similar DNase I footprint, or that the region of the protein N terminal to the zinc fingers that is necessary for binding participates in sequence-specific interactions. Alternatively, the recognition rules for GC-rich binding sites may differ from those of relatively AT-rich binding sites.

Possible roles for PRDI-BF1 zinc fingers 3 through 5. Because the last three zinc fingers of PRDI-BF1 are neither necessary nor sufficient for the recognition of PRDI, it is of interest to know what their function is. Perhaps they are involved in nonspecific DNA binding or, like the second zinc finger, can work in conjunction with the first zinc finger to contribute to sequence-specific recognition. Perhaps they influence the quality of the interaction between PRDI-BF1 and PRDI in a way that is not detectable in our assay. Alternatively, it is possible that the last three zinc fingers function in roles other than the interaction with DNA. Berg (5) has proposed that the sixth zinc finger of TFIIIA has a unique role as a linker connecting the N- and C-terminal groups of fingers. According to his model for TFIIIA binding to the 5S RNA gene internal control region, the sixth zinc finger, unlike the other eight zinc fingers that are involved in sequence-specific interactions with DNA, lies outside the major groove completely.

By combining the first two zinc fingers of PRDI-BF1 and the two zinc fingers of PRDII-BF1, we created a protein with four contiguous zinc fingers that bound specifically to both PRDI and PRDII. Although we do not know whether this protein can bind both sites at the same time or even in the same conformation, it is clear that a protein with multiple zinc fingers can use subsets of its zinc fingers to recognize distinct binding sites. Perhaps the last three zinc fingers of PRDI-BF1 recognize an RNA sequence or a DNA sequence other than that of PRDI that is not yet known. It should be possible to address this question by using recently developed methods for determining unknown target sequences of DNA-binding proteins (20, 32, 45).

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